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Honeycomb Films of Biodegradable Polymers for Tissue Engineering

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ABSTRACT

We report that microporous films (honeycomb films) can lead various types of cells to tissue formation. The honeycomb films were fabricated by applying a moist air to a spread polymer solution containing biodegradable polymers (poly(L-lactic acid) (PLLA) and poly(ε-caprolactone) (PCL)) and an amphiphilic polymer. Hepatocytes were cultured on a self-supporting honeycomb film of PLLA. The hepatocytes formed a single layer of columnar shape cells with a thickness of 20 μm. The tissue formation of hepatocytes specifically occurred on the honeycomb film of PLLA, not on a flat film of PLLA. Three dimensional tissue structures were formed, when cells were cultured on both sides of the self-supporting honeycomb film. Double layers of hepatocytes were obtained by the method. Striated tissues such as heart and blood vessel could be reconstructed by utilizing a stretched honeycomb film of PCL.

INTRODUCTION

The tissue engineering is a technology for reconstruction of living tissues. Various matrices such as gels and porous materials have been developed to realize ideal tissue formation [1]. Matrix surface, so called "bio-interface" is an important place where cells are initially attached, because the cell-matrix interaction significantly influences the subsequent cell-cell interactions [2]. In this sense, the bio-interface should be designed to cause proper cell adhesion. It is well known that surface chemistry and surface morphology are two major points to note for the design of bio-interface [3-5]. The bio-interface to be issued in this report is a microporous film of degradable polymers. Honeycomb films are microporous films of polymers which are formed spontaneously by evaporating a polymer solution in a humid atmosphere [6]. We report the honeycomb films of degradable polymers, the control of cell spreading and cell alignment on the honeycomb films, and the application of the honeycomb films to three-dimensional cell culture system.

EXPERIMENTAL DETAILS

Fabrication of honeycomb films

Honeycomb films are formed, when moist air (75% r.h. at 20°C) is applied to a droplet of polymer solution spread onto water surface. Solutions containing 1 g/L of degradable polymers ((poly(L-lactic acid): Sigma, (PLLA: Figure 1 (a)) and poly(ε-caprolactone): Birmingham Polymers, Inc., (PCL: Figure 1 (b))) and 0.1 g/L of an amphiphlic polymer (Figure 1 (c)) were prepared for the film fabrication. Benzene was utilized as a solvent for the amphiphilic polymer and PCL. Chloroform was utilized as a solvent for PLLA. One hundred μL of the

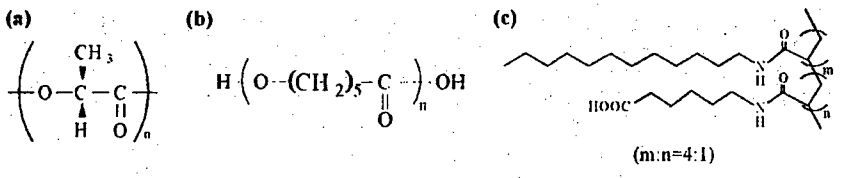

Figure 1 Polymers used for the preparation of honeycomb films. (a) poly(L-lactic acid) (PLLA). (b) poly(ε-caprolactone) (PCL). (c) amphiphilic polymer.

polymer solution was spread onto the water surface in a ϕ9 cm petri-dish and evaporated by blowing the moist air at 1200 mL/min. A polymer film floating on the water surface was transferred onto a hole that was punched in a 15 mm × 15mm of Teflon plate. Honeycomb films of PCL were stretched uniaxially with the both ends of the floating films gripped by a pair of tweezers. The honeycomb films were observed by scanning electron microscopy (S-3500N, Hitachi). For cell culture experiment, the honeycomb films were sterilized by exposing to ethylene oxide gas at 40°C.

Cell culture experiment

Hepatocytes (HEPs) were isolated from rat liver (male Wistar rats of 8 weeks old; Japan SLC, Inc) by modified Seglen's perfusion method [7]. HEPs were cultured with a Williams'E medium containing dexamethasone (1 μM, Sigma), ascorbic acid (0.28 mM, Sigma), insulin (0.57 mg/L, Sigma), epidermal growth factor (0.02 mg/L, Sigma), gentamicin (48 mg/L, Schellingplau), and aprotinin (5000 KIU/L, Sigma). Cardiac myocytes (CMYs) were isolated by enzyme treatment of minced heart tissues of 19-day rat embryos (Sprague Dawley rats; Japan SLC, Inc) [8]. CMYs were cultured with a Hepes-buffered Hams F10 medium containing 0.5 % insulin-transferrin-selenium-X (Gibco) and 3 % fetal calf serum (Gibco). Bovine aortic endothelial cells (ECs) and smooth muscle cells (SMCs) were purchased as cryopreserved samples from BioWhittaker. After the frozen cells were thawed at 37°C, the cells were resuspended into a supplemented culture medium (SmGM-2; BioWhittaker). ECs and SMCs were cultured with the supplemented medium. HEPs were cultured on a one side or both sides of honeycomb film and flat film of PLLA at the density of 1.0×10^5 cells/cm^2. CMYs were cultured on both sides of a stretched honeycomb film of PCL at the density of 1.0×10^5 cells/cm^2. ECs and SMCs were co-cultured by seeding SMCs on one side of a stretched honeycomb film and then seeding ECs on another side of the film at 6 hr after the initial plating of SMCs. The initial cell density was 2.0×10^4 cells/cm^2.

Fluorescent labeling of cells

Filamentous actin of HEPs and CMYs was stained by rhodamine-conjugated phalloidin (Molecular Probes) after fixation with paraformaldehyde (Sigma) and treatment with Triton X-100 (Sigma) at 20°C. Von Willebrand factor of ECs and α smooth muscle actin of SMCs were stained by immunological method using primary antibodies (rabbit anti-Von Willebrand factor IgG; DAKO and mouse anti-α smooth muscle actin IgG; Sigma) for each antigen and fluorescence labeled secondary antibodies (fluorescein labeled goat anti-rabbit IgG (Cappel) and rhodamine labeled goat anti-mouse IgG (Cappel)). For immunostaining, cells were fixed by

immersing into cold methanol (-20°C) for 10 min and permiabilized with 0.1% Triton X-100 for 5 min at 20°C. Fluorescence images of cells were taken by confocal laser scanning microscope (FV300; Olympus). Projected areas of cells were measured using computerized image analysis (image-pro Plus ver. 4.0; Media Cybernetics).

DISCUSSION

Self-supporting honeycomb films for cell culture substrates

Figure 2 (a) shows the scanning electron microscope (SEM) image of a honeycomb film of poly(L-lactic acid). This film has some structural features: (1) micropores with diameter of several micrometer, (2) hexagonal arrays of the micropores, (3) single layer of the array with several micrometer thickness. The honeycomb film was prepared by applying moist air to a spread polymer solution on the water surface. The porous structure is molded from the two dimensional array of water microspheres. The microspheres are formed by condensation of moist air on the surface of the spread polymer solution. The microspheres are prevented from fusing by the surfactant effect of the amphiphilic polymer.

The reasons for choosing the honeycomb film as cell culture substrates are followings: firstly, cell behavior can be controlled by porous morphology and down sized cell adhesive sites, secondly, cells can be cultured on both sides of the film, thirdly, this three dimensional culturing enables cells to interact with each other laterally on the film and also vertically via porous structure. The cell alignment is one of the typical features observed in some tissues such as muscles and blood vessels. In case of using a honeycomb film, cells cannot find a specific direction for their movement on the honeycomb film, because the honeycomb film exhibits the isotropic hexagonal pattern. An idea for the cell orientation is that a honeycomb film of an elastic polymer can be stretched and anisotropic arrays of micropores can be formed. For preparation of an elastic honeycomb film, poly(ε-caprolactone), PCL was applied. The PCL exhibits elastic property at around 20°C [9]. Therefore a self-supporting honeycomb film of PCL can be stretched at this temperature. After uniaxial stretching, arrays of elongated micropores were observed in the stretched honeycomb film shown in figure 2 (b). The arrays of the stretched micropores are applicable to guiding cell alignment.

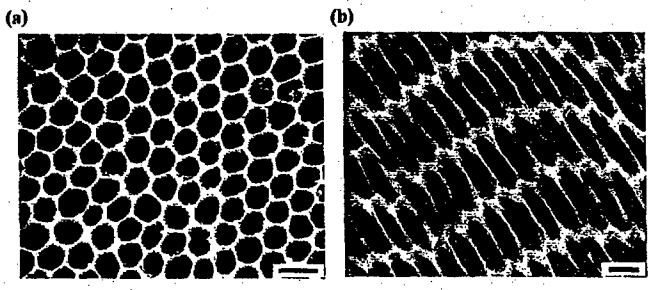

Figure 2 Scanning electron microscope images of (a) self-supporting honeycomb film of PLLA and (b) self-supporting stretched honeycomb film of PCL. Scale: 5 μm.

Cell adhesion to the honeycomb film

D. Ingber found that the total area available for cell adhesion is a key factor governing the cell spreading [10]. For the cell culture experiments, they utilized discrete micropatterns where cell adhesive islands are stamped in nonadhesive region. Our honeycomb film can be regarded as continuous micropattern of cell adhesive region. The honeycomb pattern gives reduced area for cell adhesion, compared with a flat cast film of the same polymer. Hepatocytes (HEPs) were cultured on a self-supporting honeycomb film (pore size: 4 μm and thickness: 2 μm) and a flat film of PLLA. A flat film of PLLA was fabricated by evaporating a PLLA solution on a glass plate. Figure 3 shows confocal laser scanning microscope images of filamentous actin of HEPs. Cell spreading was considerably restricted on a self-supporting honeycomb film. On a flat film, HEPs formed stress fibers of actin filaments and flat shape with thickness of less than 6 μm (Figure 3 (a): top view of cells and cross sectional image (bottom)). The average size of HEPs was 2000 μm^2/cell. On the other hand, HEPs did not form stress fibers when they were attached to the honeycomb film. The average cell size of HEPs was 900 μm^2/cell on a honeycomb film. Although cell detachment was observed on a flat film at day 5 of the culture, HEPs were not detached from a honeycomb film even at 2 weeks of the culture. On a honeycomb film, the HEPs formed a single layer of columnar shape cells with a thickness of 20 μm (Figure 3 (b): bottom). The cellular aggregates of HEPs are quite resemble to the tissue structure of liver in vivo where HEPs are adhered to adjacent cells each other via cell adhesion proteins. The tissue formation of hepatocytes specifically occurred on the honeycomb film of PLLA, not on a flat film of PLLA. The artificial tissue of HEPs secreted albumin several times more than the HEPs cultured on the flat film (data not shown).

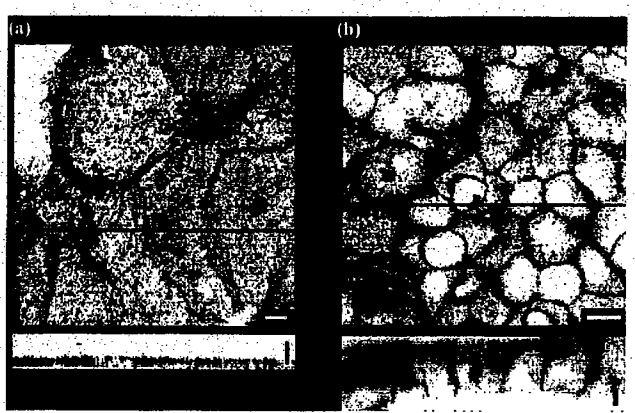

Figure 3 Difference in cell shape of HEPs: (a) on a flat film and (b) on a honeycomb film of PLLA. Cross sectional images (bottom) were obtained along the line depicted in each X-Y plane image (top). Scale: 10 μm.

Three dimensional culture system for tissue formation

The self-supporting honeycomb film is a two sided substrate. Based on the feature of the honeycomb film, we expect that cells can be cultured on both sides of the film and they can contact with each other laterally and also vertically through the micropores. In order to prove this hypothesis, various types of cells were cultured on both sides of a honeycomb film. At first, cardiac myocytes (CMYs) were cultured on both sides of a honeycomb film of PLLA. For comparison, a flat film was utilized for culture substrate of CMYs. Cardiac contraction was observed at the day 7 of the cultures. On a flat film, contraction rhythm of CMYs was random. This indicates that cell-cell contact is insulated by the polymer film. On a honeycomb film, CMYs contracted in a synchronized rhythm. This suggests that the vertical contacts of CMYs are achieved through the micropores of a honeycomb film.

The three dimensional cell culture system can be applied to the various cell types such as hepatocytes, endothelial cells, and smooth muscle cells. Figure 4 (a) shows that liver HEPs form layer structure with a thickness of 20 μm at each side of a honeycomb film. In case of using a stretched honeycomb film, CMYs are attached to both sides of the film and aligned along the stretching direction of the honeycomb film (Figure 4 (b)). Even co-culture system was established by culturing endothelial cells and smooth muscle cells on a stretched honeycomb film. Figure 4 (c) shows that each cell type was adhered separately onto each side of a stretched honeycomb film and aligned along the arrays of micropores. Heterotypic cell-cell interaction is expected in the artificial multicellular tissue.

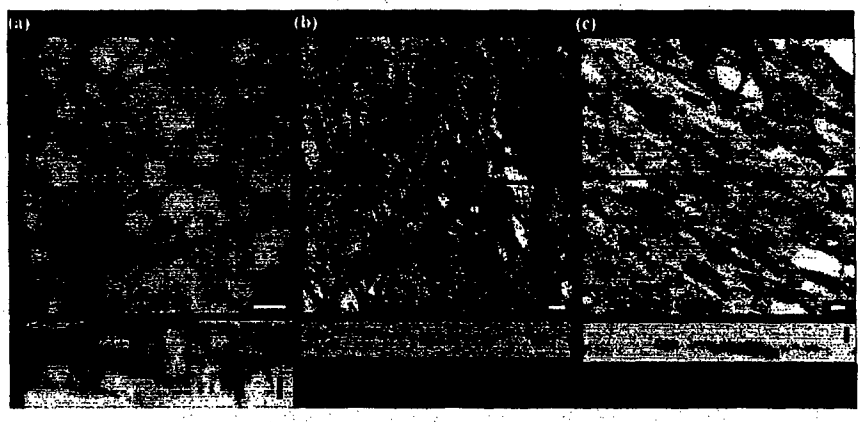

Figure 4 Three-dimensional culture of (a) HEPs, (b) CMYs, and (c) ECs (bright) and SMCs (dark) by utilizing both sides of porous films for cell attachment. The images were taken by confocal laser scanning microscopy. Cross sectional images (bottom) were obtained along the line depicted in each X-Y plane image. Scale: 10 μm.

CONCLUSIONS

In the present study, we focused on the application of microporous films to the cell culture substrates for tissue engineering. The microporous films (honeycomb films) of degradable polymers were fabricated by evaporating a polymer solution in a humid atmosphere. Anisotropic arrays of micropores were formed by stretching uniaxially a honeycomb film of an elastic polymer. Microporous surface of the honeycomb film suppressed the spreading behavior of hepatocytes. Hepatocytes on a honeycomb film formed columnar shape and layered aggregate expressing higher secretion level of albumin. The honeycomb films could be utilized for three dimensional cell culture systems. Double layered cellular aggregates were formed on the honeycomb films from hepatocytes and cardiac myocytes respectively. Even multicellular tissues like a blood vessel wall could be reconstructed by co-culturing endothelial cells and smooth muscle cells on a stretched honeycomb film. The anisotropic arrays of the stretched micropores worked as a micropattern for guiding cell alignment. As a result, the honeycomb films of degradable polymers can lead basic cell behavior, such as cell adhesion, cell movement, and cell-cell interaction to a correct way of tissue formation.

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